

# THE IN VITRO EFFECT OF PARA-AMINOSALICYLIC ACID (PAS) IN PREVENTING ACQUIRED RESISTANCE TO STREPTOMYCIN BY MYCOBACTERIUM TUBERCULOSIS

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Streptomycin was reported as being bacteriostatic and moderately bactericidal for *Mycobacterium tuberculosis* by Schatz and Waksman (1944). These results were confirmed and extended by Youmans (1945), who reported that the drug was bacteriostatic at concentrations of 0.095 to 0.28 units per ml and bactericidal at concentrations in excess of 50 units per ml. Additional confirmatory observations have been reported (Smith and McClosky, 1945; Emmart, 1945; Wolinsky and Steenken, 1947).

Youmans, Williston, Feldman, and Hinshaw (1946) and later Middlebrook and Yegian (1946) have shown that mycobacteria develop resistance to streptomycin *in vitro*. Youmans (1946) and Vennesland, Ebert, and Bloch (1948) have demonstrated that streptomycin-resistant strains are sensitive to *para*-aminosalicylic acid (PAS), the antitubercular activity of which was first described by Lehmann (1946), and later confirmed by Youmans (1946) and Sievers (1946). Streptomycin-resistant strains from tuberculous patients treated with this antibiotic have been isolated (Youmans *et al.*, 1946; Youmans and Karlson, 1947; Youmans and Feldman, 1946; Sadusk and Swift, 1947; Pyle, 1947) and when inoculated into susceptible animals produced infections refractory to streptomycin therapy (Youmans and Williston, 1946; Steenken and Wolinsky, 1948).

The principle of combined therapy was recently revived by Ungar (1943), who demonstrated the synergistic action of *para*-aminobenzoic acid and sulfapyridine with penicillin upon bacteria. Carpenter, Bahn, Ackerman, and Stokinger (1945) observed that *Neisseria gonorrhoeae* exposed to a combination of four antibacterial agents failed to develop resistance, whereas resistance developed regularly on exposure to each drug alone. Other investigators (Middlebrook and Yegian, 1946; Alexander *et al.*, 1946; Kolmer, 1947; Franks, 1946; Klein and Kimmelman, 1947) have reported on the advantages of combined therapy both *in vitro* and *in vivo*. The activity of PAS against *M. tuberculosis* when combined with streptomycin *in vitro* has been reported to be additive (Vennesland, Ebert, and Bloch, 1948). An additive action was also obtained when the drugs were used in combination in animals infected with human tubercle bacilli (Youmans, Youmans, and Osborne, 1947; McCloskey, Smith, and Frias, 1948). To supplement these observations, it was considered advisable to study what effect PAS might have upon the rate of induced resistance of the tubercle bacillus to streptomycin *in vitro*.

## MATERIALS AND METHODS

*M. tuberculosis*, human type, strain H37Rv,<sup>1</sup> was used as the test organism; the strain had been maintained by monthly passage on Petragnani's medium. All tests were performed in sorbitan monooleate albumin medium prepared as described by Dubos and Davis (1946).

Streptomycin hydrochloride (Merck) was used in this study since, at high concentrations, this form of streptomycin did not produce a precipitation in the test medium such as that observed when streptomycin calcium chloride complex was utilized. The streptomycin was dissolved in distilled water at high concentrations and sterilized by Seitz filtration. Sodium *para*-aminosalicylate was dissolved in distilled water and the solution sterilized by Seitz filtration. Appropriate dilutions and combinations of streptomycin and PAS were made in Dubos' medium.

The inocula for all tests were standardized in 19-by-150-mm cuvettes to a density permitting 89 to 91 per cent transmission of light at 620 m $\mu$  with a Coleman (model 6A) spectrophotometer. This suspension was diluted onefold, and 1 ml of the dilution was used as inoculum for each tube containing 9 ml of drug medium. The final inoculum contained approximately  $5 \times 10^6$  to  $5 \times 10^7$  viable cells as determined by the tube dilution method. The inhibiting concentration of the drug or drugs was taken as the lowest concentration that produced complete inhibition of growth after an incubation period of approximately 16 days at 37 C.

Resistance was developed by transfer of the culture that had grown to the required turbidity (89 to 91 per cent transmission), in the maximum amount of drug, to a fresh series of tubes containing graded concentrations of the drug. By observing the results of such experiments the rate of development of resistance by *M. tuberculosis* could be studied.

## RESULTS

The initial sensitivity of *M. tuberculosis* (H37Rv) to streptomycin, under these conditions, was 0.8 units per ml. Repeated exposure to the antibiotic caused a slight increase in resistance, approximately threefold, during the first 30 days (figure 1). Subsequent exposure produced a rapid increase in resistance to a level greater than 20,000 units per ml over a period of 80 days. This level represented an increase in resistance of at least 2,500-fold.

When the test was repeated, results differing only slightly from those originally obtained were observed. Resistance developed at the same rate during the first 30 days of exposure, then increased at a rate somewhat slower than in the previous experiment. The final inhibiting concentration was greater than 10,000 units per ml after a total of 120 days of exposure.

Under our test conditions the parent strain of *M. tuberculosis* was inhibited by 1.0 mg per ml of PAS. This concentration is considerably higher than that reported by other investigators (Youmans, 1946; Vennesland, Ebert, and Bloch,

<sup>1</sup> Obtained from Dr. W. Steenken, Jr., of the Trudeau Foundation, Saranac Lake, New York.

1948; Sievers, 1946) and can probably be explained on the basis of the size of the inoculum. Based on tests in which a given number of organisms is used, a 10-fold increase in the number of bacterial cells has been reported to require a 100-fold increase in the quantity of PAS needed to inhibit growth (Vennesland, Ebert, and Bloch, 1948). Exposure of the tubercle bacilli to PAS for a period of 120 days failed to produce an increase in their resistance to this antitubercular agent. In this respect the effects of PAS, when used alone, differed considerably from the results obtained with streptomycin.

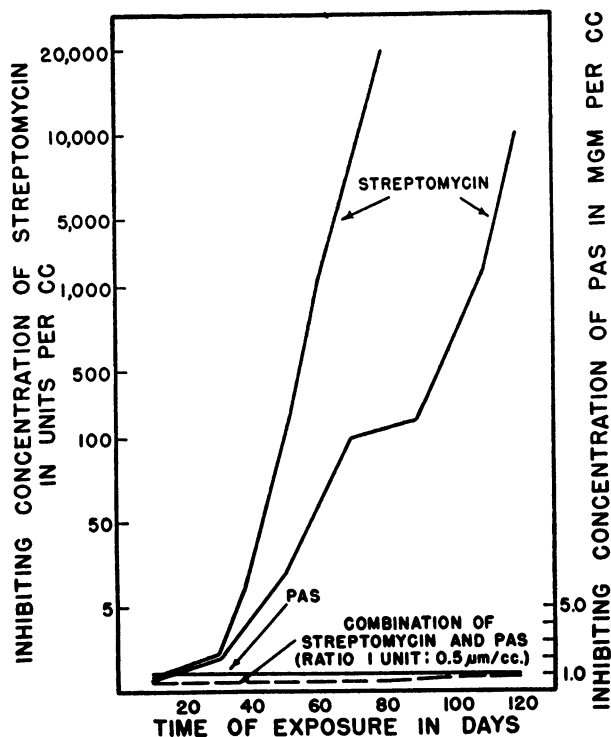


Figure 1. Induced *in vitro* resistance of *M. tuberculosis* (H37Rv) to streptomycin, PAS, and a combination of streptomycin and PAS. The results of the experiment using combined exposure are presented on the basis of the streptomycin content.

When the sensitivity of the parent culture of H37Rv was determined in the presence of both streptomycin and PAS, it was observed that a combination of 0.6 units of streptomycin plus 0.3 micrograms of PAS per ml inhibited growth. Repeated exposure, maintaining the same ratio of drug concentration throughout and using as inoculum the tube containing the maximum concentration in which the required growth had occurred, failed to produce any great change in resistance. At the conclusion of 120 days of exposure to the combined action of the drugs, the culture was inhibited by 1 unit of streptomycin plus 0.5 micrograms of PAS per ml. It is noteworthy that the concentration of PAS used in combination with streptomycin represents only 1/2,000 of the inhibiting concentration of this substance when used alone.

Since the possibility existed that the streptomycin-resistant variants were being inhibited by the PAS in the combination, it was considered of interest to determine whether the strain that had been exposed to the action of both drugs subsequently retained its sensitivity to streptomycin or to PAS when used singly. The results of these experiments indicated that the culture previously exposed to the combination was thereafter sensitive to streptomycin when used alone, since inhibition of growth occurred at a concentration of 0.8 units per ml. *Para*-aminosalicylic acid used alone was also effective and inhibited growth at 0.5 mg per ml. It was therefore apparent that the combination of PAS and streptomycin had prevented the development of streptomycin resistance. These results are presented in table 1.

The streptomycin-resistant strain of H37Rv was tested for sensitivity to PAS and found to be inhibited by 0.5 mg per ml. These results confirm the conclusions of other investigators (Sievers, 1946; Youmans, Youmans, and Osborne, 1947) that streptomycin-resistant tubercle bacilli are sensitive to PAS. The

TABLE 1  
*The action of para-aminosalicylic acid in preventing the development of resistance to streptomycin*

STRAIN	DRUG	INITIAL SENSITIVITY (CONCENTRATION PER ML)	NO. OF DAYS EXPOSED	INHIBITING CONCENTRATION AFTER EXPOSURE	
				Streptomycin units per ml	PAS mg per ml
H37Rv	Streptomycin	0.8 units	120	10,000-20,000	0.5
H37Rv	PAS	1.0 mg	120	0.8	1.0
H37Rv	Streptomycin plus PAS	0.6 units 0.3 microgram	120	0.8	1.0

bacterial strain that was exposed to PAS and failed to increase in resistance to this agent was found to have the same sensitivity to streptomycin as the unexposed parent strain.

#### DISCUSSION

The development of drug fastness during streptomycin therapy in tuberculosis causes the administration of the antibiotic thereafter to be of questionable value. Since the degree to which resistance occurs does not permit compensation by correspondingly increased dosage, any relatively nontoxic agent that would reduce or prevent the development of streptomycin resistance would probably improve the clinical value of the antibiotic. PAS appears to be such an agent. This compound at relatively low concentrations appears to offer promise as an agent that might prevent *in vivo* the development of resistance to streptomycin by the tubercle bacillus. When used in such a way as to maintain high concentrations, it may exert a therapeutic action in human tuberculosis when resistance to streptomycin has developed. Since streptomycin resistance is rarely induced in animals, it is difficult at present to test this hypothesis by animal experimentation.

The results of these *in vitro* experiments suggest the advisability of determining whether the administration of PAS with streptomycin in the treatment of human tuberculosis will prevent the development of streptomycin resistance or effect a satisfactory clinical improvement once resistance has occurred.

#### SUMMARY

*Mycobacterium tuberculosis*, strain H37Rv, develops resistance to streptomycin *in vitro* when exposed to increasing concentrations of the antibiotic.

The streptomycin-resistant strain of H37Rv retained its sensitivity to PAS.

Repeated exposure of *M. tuberculosis* to PAS for 120 days failed to produce an increase in the resistance of this strain.

The addition of relatively low concentrations of PAS to streptomycin (approximately 0.5 micrograms to 1 unit) prevents or greatly retards the *in vitro* development of streptomycin resistance.

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